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| Original Article |

# Effects of Desflurane-Induced Preconditioning Following Ischemia-Reperfusion on Modulation of Calcium Homeostasis in Rat Heart

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**Abstract :** $Ca^{2+}$ overload induced by ischemia-reperfusion alters $Ca^{2+}$ homeostasis, which plays an important role in myocardial cell injury. Although desflurane has been commonly used in anesthesia, little is known about its cardioprotective effect associated with $Ca^{2+}$ homeostasis in ischemia-reperfused rat heart. Artificially ventilated anaesthetized Sprague-Dawley rats were subjected to a 30 min of left anterior descending coronary artery occlusion followed by 2 h of reperfusion. Rats were randomly assigned to one of three groups; Sham, I/R only, desflurane preconditioning group. In the present study, desflurane reduced infarct size (43.6±5.5% vs. 19.1±1.9% for I/R and desflurane, respectively, $p<0.01$). In desflurane-treated rat heart, we observed a consistent decrease in the expression of pro-apoptotic protein Bax leading to a decrease in cytochrome c release. We also found that desflurane enhanced expression of anti-apoptotic protein Bcl-2, activated ERK concerned with survival, and significantly attenuated abnormal changes of sarcoplasmic reticulum genes and proteins in ischemia-reperfused rat heart. These results suggest that desflurane prevents myocardial injury in response to ischemia-reperfusion by modulating sarcoplasmic reticulum function.

**Key words:** *Desflurane, $Ca^{2+}$ homeostasis, ischemia-reperfusion*

## 1. Introduction

Ischemia-reperfusion (I/R) injury in myocardium triggers stress signaling processes that eventually results in cell death and myocardial damage.[1,2] A proposed underlying mechanism include intracellular $Ca^{2+}$ overload as a consequence of dysregulation of $Ca^{2+}$ homeostasis, which has been proposed as an important therapeutic target for increasing the tolerance to I/R injury.[3] In attempt to lessen the I/R injury, ischemic preconditioning has been introduced as an effective treatment modality.[4] Ischemic preconditioning is an endogenous phenomenon whereby repeated brief episodes of coronary artery occlusion protect the heart against further prolonged ischemia.[5] Interestingly, it has been shown that certain anesthetic agents such as volatile anesthetics and opioids possess cardioprotective effects against I/R injury, mimicking ischemic preconditioning.[6-9] Anesthetic agents appear to prevent the accumulation of intracellular $Ca^{2+}$ that occurs during myocardial hypoxia or ischemia and may induce cell apoptosis leading to cardiomyopathy and heart failure.[10-12] Therefore, the interruption of $Ca^{2+}$ overload has been proposed as an important target for increasing tolerance to I/R injury.

Volatile anaesthetics induce cardioprotective effects through signal transduction pathways similar to those observed during ischemic preconditioning such as activation of protein kinase C, mitochondrial adenosine triphosphate-regulated potassium channels, and reactive oxygen species.[13-15] Among volatile anesthetic agents, desflurane has been shown to confer cardioprotection against I/R injury, which mimics ischemic preconditioning effects.[16-21] However, evidence is lacking regarding I/R injury associated with $Ca^{2+}$ homeostasis. The aim of this study was to determine whether desflurane protects myocardium against I/R injury by regulating $Ca^{2+}$ homeostasis by measuring myocardial infarct size, the expression of proteins involved in survival signals, apoptosis, and the expression of genes and proteins influencing $Ca^{2+}$ homeostasis.

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## 2. Materials and Methods

### 2.1 Animal Preparation

Animal experimental procedures were approved by the committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine and were performed in accordance with the Committee's Guidelines and Regulations for Animal Care. Male Sprague-Dawley rats (250-300 g) were anesthetized with a single i.p. injection of pentobarbital 60 mg $kg^{-1}$. After tracheal intubation, the rats were artificially ventilated (Harvard Apparatus 683, MA, USA) with oxygen 100%. A heparinized catheter was inserted into the right femoral artery for continuous monitoring of mean aortic pressure, and a Lead-II electrocardiogram monitored heart rate via subcutaneous stainless steel electrodes connected to a PowerLab monitoring system (ML845 PowerLab with ML132; AD Instruments, CO, USA). The tail vein was cannulated to infuse saline. Rectal temperature was maintained at 38°C during the entire experiment using a heating pad connected to a rectal probe.

### 2.2 Experimental Protocol

After a left thoracotomy and pericardiotomy, the heart was exposed and a suture was passed around the left anterior descending (LAD) coronary artery by inserting a small curved Prolene 6.0 needle into the margin of the pulmonary cone, exiting through the middle of a line linking the cone to the atrium. The suture ends were threaded through a small vinyl tube to prepare a snare. The LAD coronary artery was occluded for 30 min by tightening the snare. Myocardial ischemia was confirmed by the appearance of a regional cyanosis on the epicardium distal to the snare and akinesia or bulging in this area. After a 30 min ischemia, the snare was released and reperfused for a period of 2 h. The thread passed around the LAD coronary artery was left in place. Hemodynamic parameters were measured at the following time points; (1) 20 min before desflurane treatment (baseline), (2) at the end of desflurane treatment (or no treatment), 3) at the end of ischemia (or no ischemia), and (4) at the end of reperfusion (2 h).

### 2.3 Study Groups

Rats were randomly assigned to one of the three treatment groups (n = 10 for each group, Fig. 1) and received 30 min of occlusion followed by 2 h reperfusion. The groups were as follows. 'I/R': rats did not receive desflurane treatment during the entire experiment; 'Desflurane': rats were subjected to inhalation of desflurane for 20 min before 30 min of occlusion followed by 10 min washout. Desflurane 5.7%, corresponding to 1 MAC in rat,[22] was delivered in oxygen (100%, flow rate: 2 L/min) through desflurane vaporizer (Devapor Type M32600, Dräger AG, Germany). The end-tidal concentrations of desflurane were monitored with a gas analyzer (Capnomac, Datex, Helsinki, Finland) that had been calibrated before use. Sham rats were treated similarly except that the coronary suture was not tied and desflurane was not administered.

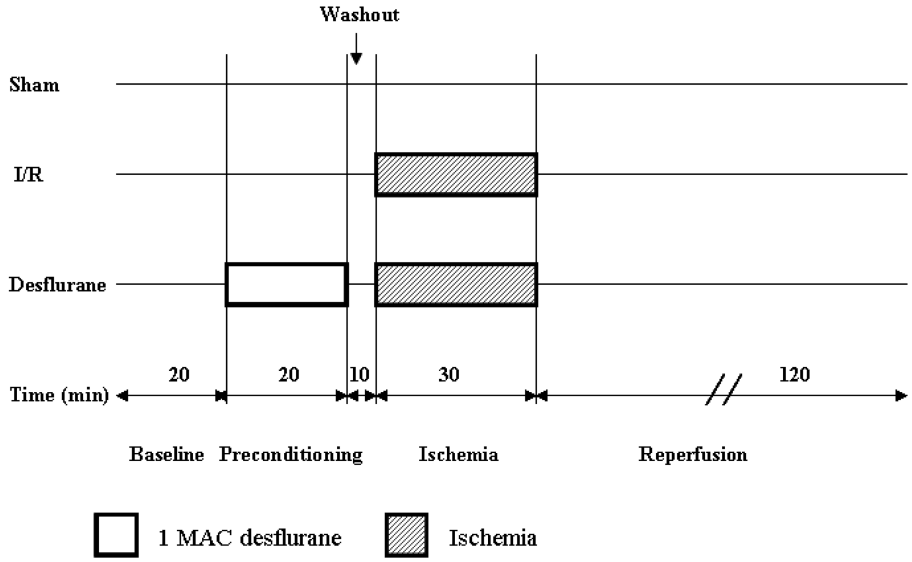


Figure 1. Occlusion of the left anterior descending (LAD) coronary artery was maintained for 30 min followed by reperfusion. After 2 h, sampling was performed for measurement of infarct size, immunoblot, and RT-PCR, respectively. I/R, ischemia/reperfusion group; Desflurane, desflurane administered during the preconditioning period; Sham, the coronary suture was not tied and desflurane was not administered. MAC: minimum alveolar concentration.

### 2.4 Measurement of Infarct Size

At the end of the 2 h reperfusion period, the heart was excised and immersed in 2% triphenyltetrazolium chloride (TTC) (Sigma Chemicals, MO, USA) for 20 min at 37°C. The infarcted myocardium, which does not take up TTC stain when the dehydrogenase enzymes are drained off, remains pale in color. The hearts were sliced and photographed, and infarct size was determined by dividing the total necrotic area of the left ventricle by the total left ventricular area.[23] The boundary of unstained area was traced in a blinded fashion and quantified with NIH image, version 1.61.

### 2.5 Immunoblot Analysis

At the end of the 2 h reperfusion period, tissue preparation was performed as previously described.[24] Briefly, tissue specimens were pulverized and dissolved in lysis buffer (Cell Signaling, MA, USA). The solution was vigorously homogenized with Pyrex Potter-Elvehjem Tissue Grinder (BLD Science, NC, USA) and centrifuged at 12,000×g for 10 min at 4°C. The supernatant was transferred to a new tube and stored at -70°C. Protein concentrations were determined using the Bradford protein assay kit (BioRad, CA, USA). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Co, MA, USA). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% non-fat dried milk for 1 h at room temperature, membranes were washed twice with TBS-T and incubated with primary antibodies for 1 h at room temperature or overnight at 4°C. The following primary antibodies were used: rabbit anti-extracellular signal-regulated kinases (ERK), mouse anti-phospho ERK, mouse anti-Bcl-2, mouse anti-Cytochrome C, goat anti-rat sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA2), mouse phospholamban (PLB), rabbit calsequestrin (CSQ), anti-ryanodine receptor (RyR), phosphorylated PLB at serine-16 (Ser), phosphorylated PLB at threonine-17 (Thr) (Santa Cruz Biotechnology, CA, USA), rabbit anti-Bax (Assay Designs, MI, USA), and mouse anti-β-actin antibodies (Sigma Chemicals, MO, USA). The membranes were washed three times with TBS-T for 10 min, and then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, bands were detected by enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology, CA, USA). β-actin gene was used as the standard for equalizing the protein samples. The band intensities were quantified using NIH image, version 1.61. Each experiment was performed at least three times.

### 2.6 RT-PCR Analysis

Tissue samples were vigorously homogenized with Pyrex Potter-Elvehjem Tissue Grinder (BLD Science, NC, USA) in TRI Reagent (Sigma Chemicals, MO, USA). Total RNA was prepared using the UltraspectTM-II RNA system(Biotecx Laboratories, Inc., TX, USA) and single-stranded cDNA was synthesized from isolated total RNA using avian Myeloblastosis virus (AMV) reverse transcriptase. A 20 μl reverse transcription reaction mixture containing 1 g of total RNA, 1X reverse transcription buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs), 0.5 unit of RNase inhibitor, 0.5 g of oligo (dT)15, and 15 units of AMV reverse transcriptase was incubated at 42°C for 15 min, heated to 99°C for 5 min, and then incubated at 0-5°C for 5 min. All primer pairs for DNA sequence of proteins related to calcium homeostasis are shown in Table 1. PCR conditions were 94°C for 3 min, 94°C for 1 min, 41-49°C for 1 min and 72°C for 2 min, 35 cycles, with a final extension for 10 min at 72°C. GAPDH gene (primers 5'-accacagtccatgccatcac-3' and 5'-tccaccaccctgttgctgta-3', 450 bp) was used as the internal standard. The signal intensity of the amplification product was analyzed using the UVlband

Table 1. The nucleotide sequence of all primer pairs for proteins related to $Ca^{2+}$ homeostasis.

| Genes | Primer sequence | Size (bp) |
|---|---|---|
| L-type $Ca^{2+}$-channel | Sense: 5- TGTCACGGTTGGGTAGTGAA-3<br>Antisense: 5'-TTGAGGTGGAAGGGACTTTG-3' | 346 |
| $Na^{+}$-$Ca^{2+}$ exchanger | Sense: 5-TGTCTGCGATTGCTTGTCTC-3<br>Antisense: 5-TCACTCATCTCCACCAGACG-3 | 364 |
| SR $Ca^{2+}$-ATPase | Sense: 5-TCCATCTGCCTGTCCAT-3<br>Antisense: 5-GCGGTTACTCCAGTATTG-3 | 196 |
| Phospholamban | Sense: 5-GCTGAGCTCCCAGACTTCAC-3<br>Antisense: 5-GCGACAGCTTGTCACAGAAG-3 | 339 |
| Ryanodine receptor 2 | Sense: 5-CCAACATGCCAGACCCTACT-3<br>Antisense: 5-TTTCTCCATCCTCTCCCTCA-3 | 351 |
| Calsequestrin | Sense: 5-TCAAAGACCCACCCTACGTC-3<br>Antisense: 5-CCAGTCTTCCAGCTCCTCAG-3 | 352 |

Table 2. Hemodynamic parameters

| | Baseline | | Preconditioning | | Ischemia | | Reperfusion | |
|---|---|---|---|---|---|---|---|---|
| | HR | MAP | HR | MAP | HR | MAP | HR | MAP |
| I/R | 435±52 | 139±6 | 434±50 | 137±9 | 444±45 | 121±10 | 451±42 | 114±6 |
| Desflurane | 425±45 | 134±8 | 401±43 | 89±9* | 457±42 | 117±6 | 439±33 | 101±9 |

Data are mean±SD. HR, heart rate (beats per min); MAP, mean arterial pressure (mmHg). I/R, ischemia/reperfusion; Desflurane, desflurane administered during the preconditioning period. * Significantly ($P$<0.05) different from baseline.

software (UVltec, Cambridge, UK).

### 2.7 Statistical Analysis

Data analysis was performed using the statistical software program Prism v3.0 (GraphPad Software, USA). Comparisons among multiple groups were performed using one-way ANOVA (Analysis of Variance) with Bonferroni's test. Data are presented as mean±SD or SEM of more than three separate experiments performed in triplicate. Statistical significance was defined as p<0.05.

## 3. Results

### 3.1 Hemodynamics and Measurement of Infarct Size

The heart rate and mean arterial pressure are summarized in Table 2. The mean arterial pressure during the preconditioning period in desflurane group was significantly lower (p<0.05) than that of the baseline value. The other values of mean arterial pressure and heart rate revealed no differences in either inter- or intragroup comparisons. Infarct sizes are shown in figure 2. Mean infarct size of the left ventricle in the I/R group was 43.6 ±5.5%. In the desflurane-treated group, infarct sizes of the left ventricle were significantly reduced to 19.1±1.9% (p<0.01).

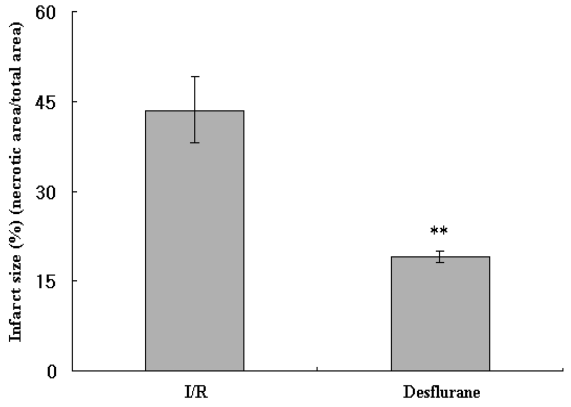


Figure 2. Infarct size was analyzed using planar morphometry in 2,3,5-triphenyltetrazolium (TTC) stained sections and expressed as a ratio of the left ventricular area. I/R, ischemia/reperfusion group; Desflurane, desflurane administered during the preconditioning period. Each value is the mean±SEM of 10 hearts/group. Differences were considered statistically significant when *p<0.05 vs. I/R group.

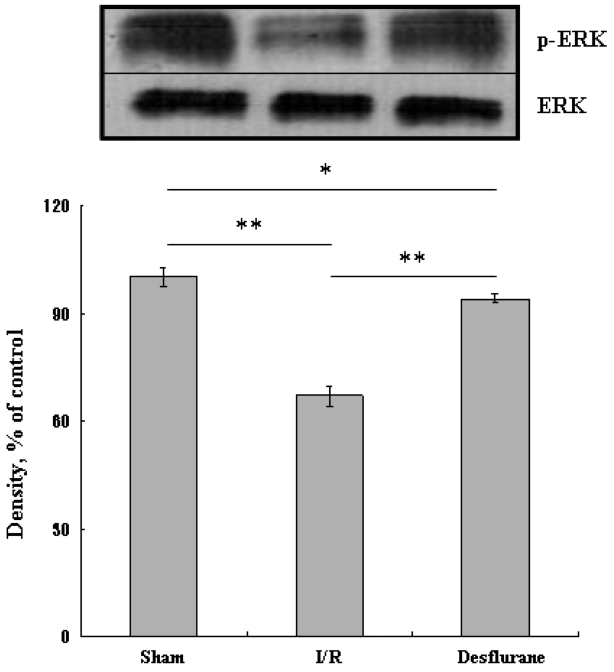


Figure 3. Western blot analysis measuring ERK and p-ERK. Each signal was quantified by scanning densitometry. I/R, ischemia/reperfusion group; Desflurane, desflurane administrated during the preconditioning period; Sham, the coronary suture was not tied and desflurane was not administered. Each value is the mean ±SEM of 10 hearts/group. Differences were considered statistically significant when *p<0.05, **p<0.01.

### 3.2 Western Blot Analysis on Phosphorylation of ERK and Apoptosis Proteins

The activation of extracellular signal-regulated kinases (ERK 1/2) plays an important role in the mechanisms of cellular survival and proliferation through gene regulation.[25] ERK 1/2 are dual specificity mitogen-activated protein (MAP) kinase and we measured phosphorylation of ERK (42 and 44 kDa) by immunoblot assay. The phosphorylation activity of ERK was dramatically lower in I/R group than in sham group. Rats treated with desflurane showed significantly higher levels of ERK activity than those in the I/R group (Fig. 3). Ischemia and

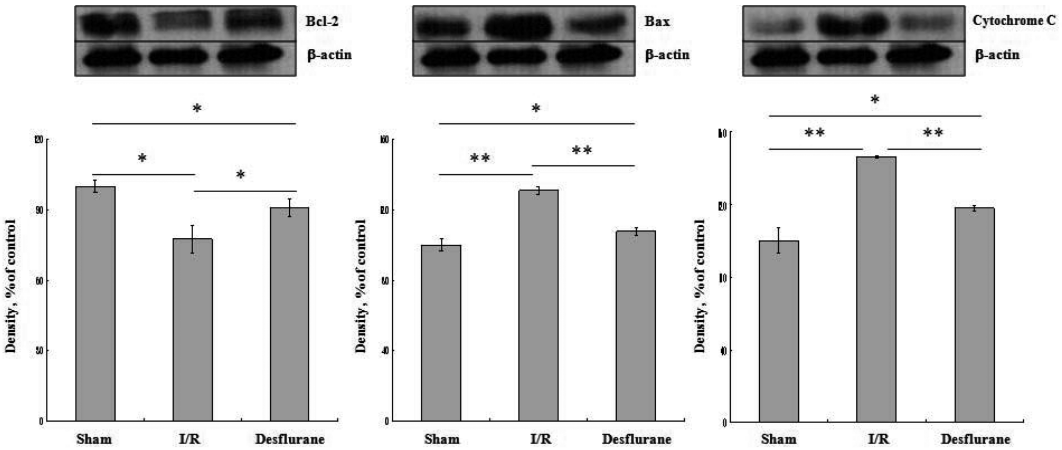


Figure 4. Western blot analysis measuring Bcl-2, Bax, and cytochrome c. Each signal was quantified by scanning densitometry. β-actin was used as an internal standard. I/R, ischemia/reperfusion group; Desflurane, desflurane administered during the preconditioning period; Sham, the coronary suture was not tied and desflurane was not administered. Each value is the mean±SEM of 10 hearts/group. Differences were considered statistically significant when *p<0.05, **p<0.01.

reperfusion increased expression of pro-apoptotic proteins such as Bcl-2-associated X protein (Bax) and cytochrome c and decreased expression of the anti-apoptotic protein, B cell leukemia/lymphoma-2 (Bcl-2) (Fig. 4). In the desflurane-treated group, the expression levels of Bax and cytochrome c were decreased, whereas the expression level of Bcl-2 was

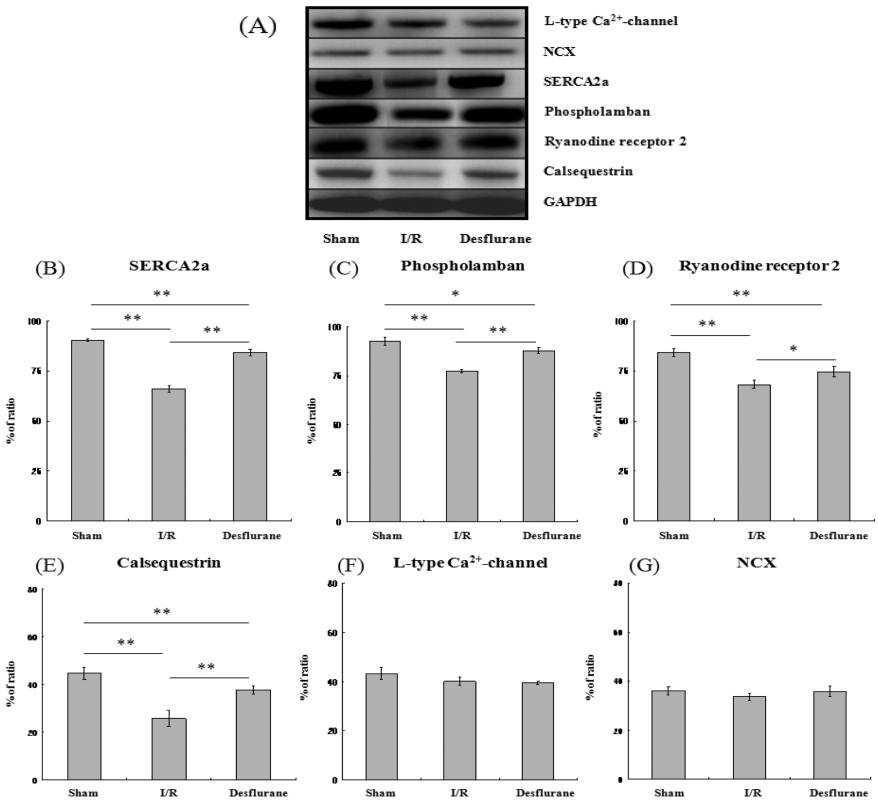


Figure 5. Analysis of the expressions of genes related to $Ca^{2+}$ homeostasis. (A), Representative gel image of mRNA levels. B-G: Histograms of SERCA2a (B), phospholamban (C), ryanodine receptor 2 (D), calsequestrin (E), L-type $Ca^{2+}$-channel (F), and $Na^{+}$-$Ca^{2+}$ exchanger (NCX) (G). GAPDH was used as an internal standard. I/R, ischemia/reperfusion group; Desflurane, desflurane administrated during the preconditioning period; Sham, the coronary suture was not tied and desflurane was not administered. Each value is the mean ± SEM of 10 hearts/group. Differences were considered statistically significant when *p<0.05, **p<0.01.

increased when compared to that of the I/R group.

### 3.3 Expression of Genes and Proteins Influencing Calcium Homeostasis

In order to assess gene expressions related to $Ca^{2+}$ homeostasis, total RNA was isolated and analyzed by semi-quantitative RT-PCR. The levels of gene expression for sarcoplasmic reticulum (SR) proteins such as SERCA2a, PLB, $RyR_2$, and CSQ were decreased in I/R rat heart, while treatment with desflurane resulted in upregulation of mRNA transcription levels for SR proteins. However, L-type $Ca^{2+}$ channels and $Na^+$-$Ca^{2+}$ exchanger (NCX) were unaltered by ischemia-reperfusion with or without desflurane administration (Fig. 5). To further confirm the functional changes of SR proteins related to alteration of mRNA expression, we examined protein levels of SERCA2a, PLB, RyR, and CSQ. Most SR proteins were decreased in I/R group but restored in the desflurane-treated group (Fig. 6). These results are in agreement with changes seen in mRNA transcription levels. Although PLB protein contents were unaltered, all phosphorylated PLB proteins normalized to total PLB were increased in desflurane-treated group (Fig. 7), indicating that PLB in I/R hearts were predominantly in the unphosphorylated form.

## 4. Discussion

In this study, we observed a significant reduction in ischemia-reperfusion induced myocardial infarct size in the desflurane-treated group. Our results demonstrated that the cardioprotective effect of desflurane occurs in association with modulation of proteins involved in $Ca^{2+}$ homeostasis. Several studies have reported that desflurane decreased infarct size in various experimental models and species.[16-18] Here, we also confirmed that desflurane-induced preconditioning dramatically reduced myocardial infarct size. Desflurane has been reported more cardioprotective than the other volatile anesthetics.[16]

ERK 1/2 are members of the mitogen-activated protein (MAP) kinase-family of serine-threonine kinases involved in regulating cell proliferation, differentiation and survival. ERK 1/2 are activated in response to ischemia-reperfusion, oxidative stress, hypoxia, and adrenergic stimulation,[25] and mediate cellular protection during ischemia-reperfusion.[26] Our results demonstrated that desflurane activates ERK and maintains viability in rat heart exposed to ischemia-reperfusion, similar to that reported previously.[21,27] In response to an apoptotic stimulus such as I/R, the pro-apoptotic protein, Bax, undergoes

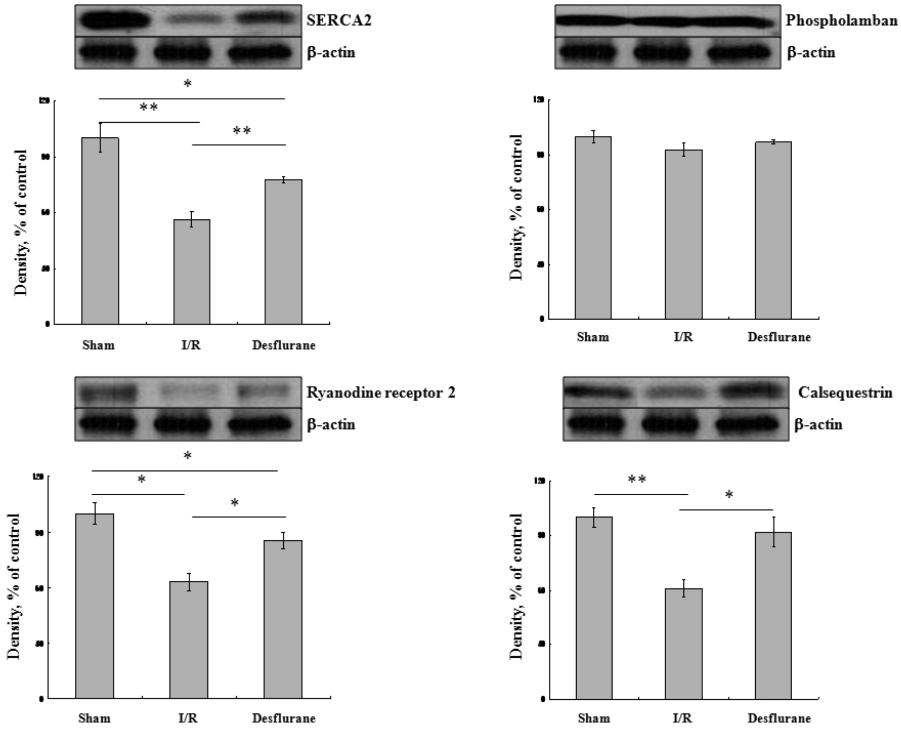


Figure 6. Western blot analysis of SR proteins. Each signal was quantified by scanning densitometry. β-actin was used as an internal standard. I/R, ischemia/reperfusion group; Desflurane, desflurane administrated during the preconditioning period; Sham, the coronary suture was not tied and desflurane was not administered. Each value is the mean±SEM of 10 hearts/group. Differences were considered statistically significant when *p<0.05, **p<0.01.

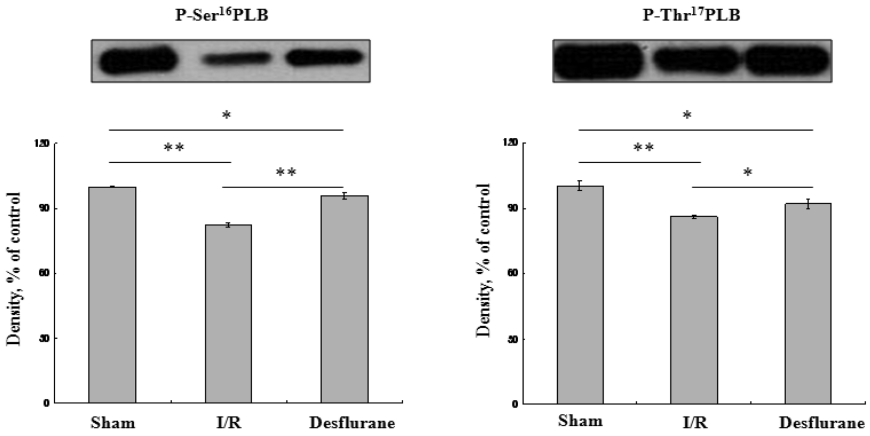


Figure 7. Western blot analysis of PLB-Ser and PLB-Thr. Each signal was quantified by scanning densitometry. Total PLB was used as an internal standard. I/R, ischemia/reperfusion group; Desflurane, desflurane administered during the preconditioning period; Sham, the coronary suture was not tied and desflurane was not administered. Each value is the mean±SEM of 10 hearts/group. Differences were considered statistically significant when *p<0.05, **p<0.01.

a conformational change that allows it to translocate to the mitochondria where it induces cytochrome c release. In addition, activation of ERK 1/2 inhibits conformational change in Bax protein and cytochrome c-induced caspase activation, thereby preventing apoptosis.[28] The anti-apoptotic protein Bcl-2 has been identified in the outer mitochondrial membrane and is known to attenuate cellular injury by inhibiting cytochrome c translocation, preventing injurious $Ca^{2+}$ release from the endoplasmic reticulum[29] and inhibiting Bax translocation from the cytoplasm to the mitochondria.[30] During ischemic preconditioning, an increase in Bcl-2 in association with a decrease in the pro-apoptotic protein Bax has been reported in isolated rat heart.[31] In the present study, we observed an increase in expression of the anti-apoptotic protein Bcl-2, a decrease in the expression of the pro-apoptotic protein Bax as well as attenuation of mitochondrial cytochrome c release, which indicate that desflurane induces a cardioprotective effect against apoptosis caused by I/R.

$Ca^{2+}$ is an important messenger in intracellular signal transduction and plays an important role in cardiac excitation-contraction coupling.[32] During the process of excitation-contraction coupling, intracellular $Ca^{2+}$ homeostasis is carefully regulated by ion channels, specific binding and transport proteins. It has been proposed that changes in $Ca^{2+}$ homeostasis play an important role in modulation of apoptosis,[33] and the balance between Bcl-2 and Bax affects mitochondrial $Ca^{2+}$homeostasis, which is important in determining whether cells survive or undergo apoptosis. Therefore, intracellular dysregulation of $Ca^{2+}$ homeostasis has been proposed as one of the mechanisms of cell injury induced by I/R.

SR plays a central role in regulating intracellular $Ca^{2+}$ concentration and contains SR $Ca^{2+}$-cycling proteins such as RyR, SERCA2a, PLB, and CSQ. The reduced expression of SR gene in I/R rat heart in our study was in accordance with a previous finding that expression of SR gene is markedly decreased in I/R.[34] We also found that treatment with desflurane restored the level of SR gene expression. In addition, our results regarding expression of SR proteins were in accordance with those seen for SR genes. These results indicate that desflurane modulates SR gene expression in ischemia-reperfused rat heart. Although the expression of SR genes was increased by antioxidants and ß-adrenergic receptor blockers,[34,35] further studies are needed to investigate the transcription mechanism regulating the expression of SR gene by desflurane.[36] Previous reports have concluded that reduced PLB phosphorylation in I/R is related to reduced SR $Ca^{2+}$ uptake,[37-39] thus, the increase in PLB phosphorylation in desflurane-treated rat may be responsible for recovery of SERCA2a activity. $Ca^{2+}$ entry across the membrane through L-type $Ca^{2+}$ channels is balanced by an efflux of $Ca^{2+}$ from $Na^{+}$-$Ca^{2+}$ exchanger (NCX).[31] In this study, we found that transcription levels of L-type $Ca^{2+}$ channels and NCX were not altered, similar to our previous study on propofol.[40] In contrast, increases in NCX expression were reported in various animal models of heart failure.[41-43] Therefore, we suggest that it may be dependent on animal species and experimental conditions.

In conclusion, desflurane confers protection against injury in ischemia-reperfused rat heart. Furthermore, changes in SR proteins related to $Ca^{2+}$ homeostasis are attenuated by desflurane, which produce a cardioprotective effect as a result

of reduction of apoptotic cell death.

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